TITRATION CURVES OF LIGANDED HEMOGLOBINS BY COMBINED ISOELECTRIC FOCUSING–ELECTROPHORESIS

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Received 7 August 1978

1. Introduction

By exploiting an original idea of Rosengren et al. [1] we have demonstrated the possibility of performing protein titration curves by electrophoresis in a stationary pH gradient stabilized by a stack of focused carrier ampholytes in the pH 3–10 range [2]. By running a protein and its genetic mutants in parallel, the shape of the respective titration curves revealed which charged amino acid had been substituted in the mutant phenotype. Moreover, at least in the case of hemoglobin, it was possible to correlate the electrophoretic mobility at a given pH with the number of protons bound or released by the macromolecule.

We have now extended our investigation to liganded states of proteins, in order to see whether the bound species could be resolved, by the present technique, from unliganded ones, and their physicochemical properties studied. We have selected as a model the interaction of hemoglobin (Hb) with organic phosphates, as these ligands are very important in regulating the oxygen affinity of Hb in mammalian [3] as well as in avian [4] red blood cells. We had predicted that, if the liganded form could be isolated, it should be possible to measure the half-life and the pH range of stability of the complex as well as the stoichiometry of the protein–ligand complex. Moreover, the shape of the relative titration curves of the liganded versus the unliganded species should allow the estimation of the amino acid(s) involved in ligand binding.

2. Materials and methods

Glucose-6-phosphate (G6P), pyridoxal phosphate (PLP), inositol hexaphosphate (IHP) and 2,3-diphosphoglyceric acid (DPG) were from Sigma. Inositol hexasulphate (IHS) was a kind gift from Drs R. and R. E. Benesch, Columbia University, NY.

Routine procedures were used for the preparation of hemolysate. Affinity labelling of hemoglobin by G6P was done according to the technique in [5]. Specific fixation of PLP on N-termini of β chains of hemoglobin and its stabilisation by reduction with sodium borohydride ($NaBH_4$) were performed as in [6]. G6P and PLP complexes were desalted through Sephadex G-25 just prior to electrophoresis.

IHP and IHS binding studies were done on MetHb in equimolar ratio whereas DPG was added in equimolar ratio to deoxyHb and then applied directly to the gel without desalting.

The two dimensional (2D) isoelectric focusing (IEF)–electrophoresis technique [2], pH measurements [7,8] and gel staining [9] were performed as described.

3. Results and discussion

Figure 1A shows the titration curves of free Hb in equilibrium with the Hb–G6P complex. It appears that the free and bound forms are in a ratio of ~7:3. Moreover, since the bound form is stable from

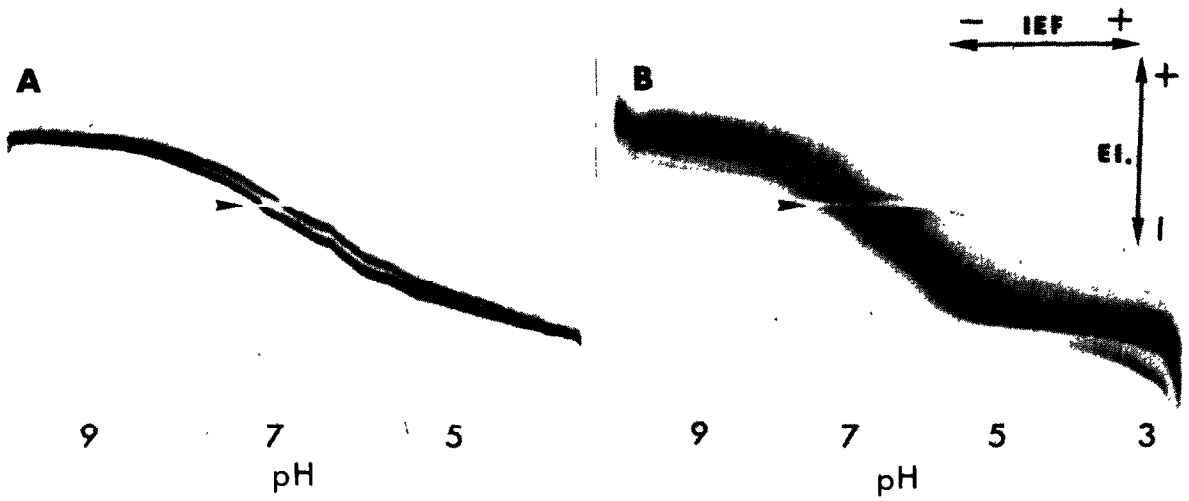

Fig.1. Titration curves of a mixture of HbA and HbA–G6P complex. Gel slab (2 mm thick): 7% acrylamide, 2% Ampholine pH 3.5–10, 5 mM Asp, 5 mM Glu and 5 mM Lys. First dimension: 90 min run in the LKB 2117 Multiphor chamber by delivering 15 W with an LKB constant wattage power supply. Coolant temperature: 2°C. Second dimension: 20 min run at 800 V, 10 mA with a sample load of ~200 μg protein. Staining: colloidal dispersion of 0.1% Coomassie brilliant blue G-250 in 12% trichloroacetic acid and 1 N H_2SO_4 as in [9]. (A) Red blood cell lysate incubated for 4 h with a 5-fold molar excess of G6P in the deoxyHb state; (B) same as (A) except that the sample was then treated with $NaBH_4$ and run for 35 min. Notice that in this case the HbA–G6P complex is now ~70% of the total. The two arrows and positive and negative symbols represent the direction and polarity of isoelectric focusing (IEF) and electrophoresis (El.). The arrowhead indicates the application zone (i.e. the zero mobility or isoelectric plane). In both cases, the joining of the two curves around pH 4 is due to instability of unliganded HbA at this pH.

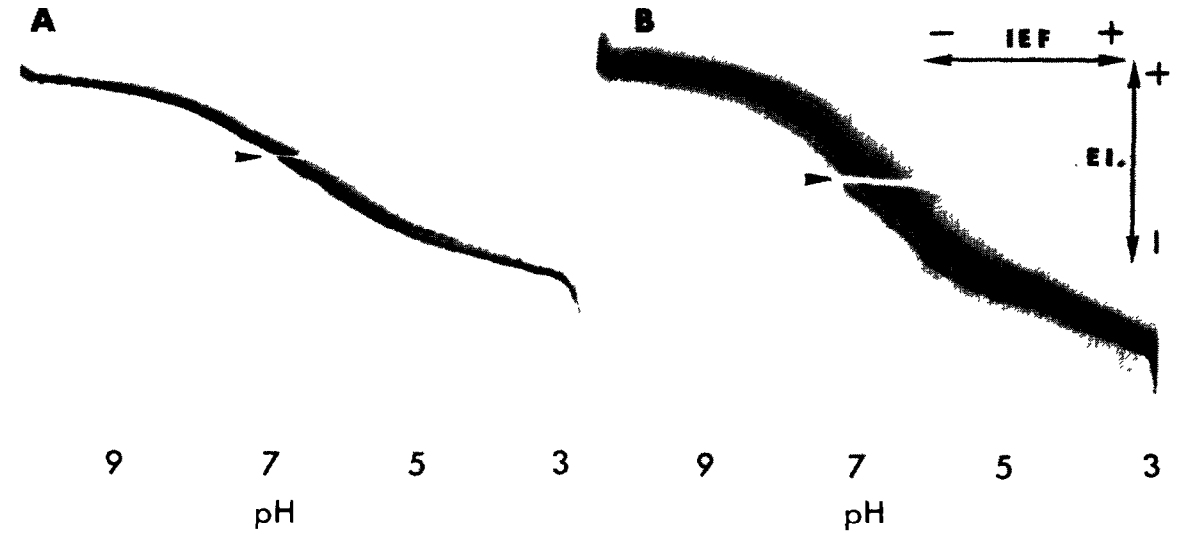

Fig.2. Titration curves of HbA versus HbA–PLP complex. (A) Red blood cell lysate incubated for 4 h with a 3-fold molar excess of PLP in the deoxyHb state. The complex is stable only in the range pH 4–8; (B) same as (A) after reduction with $NaBH_4$. Notice that now the complex is stable in the range pH 3.5–10 and that at least 3 major bands can be distinguished. All other conditions as in fig.1.

pH ~3.5–10 for all the period of electrophoresis (20 min) in the absence of excess ligand and in presence of O_2, we have concluded that G6P has to be covalently bound to the protein moiety. This is in agreement with [5] and also with the fact that reduction with $NaBH_4$ does not change the picture (fig.1B) except for shifting the equilibrium toward the G6P complex, which now appears to be ~70% of the total (the reduction was performed in presence of a 5-fold molar excess of G6P).

Figure 2A shows the titration curves of free Hb versus the Hb–PLP complex. The two curves are only split in the region pH ~4–8, indicating that the complex is stable only in this pH range. For the same reasons outlined above, it is reasonable to assume that PLP is covalently bound to Hb, but via an unstable link (e.g., an aldimine) which readily splits outside this pH range. The formation of unstable aldimine bonds (Schiff's bases) is a well known mechanism for the action of PLP in enzymatic reactions. This was confirmed by reduction with $NaBH_4$, with concomitant formation of a stable secondary amine bond.

As shown in fig.2B, three major components can now be seen in the region pH 3.5–10, which should correspond to free Hb, to mono-substituted tetramer $\alpha_2\beta\beta^{PLP}$ and to disubstituted tetramer $\alpha_2(\beta^{PLP})_2$ as demonstrated [6,10,11]. The two minor zones could correspond to similar PLP binding to α subunits, since there are always traces of oxyHb during incubation.

Figure 3A depicts the titration curves of free MetHb versus the MetHb–IHP complex. IHP is known to fit in a cavity between the two β subunits in deoxyHb, i.e. in the same binding site of DPG, and to form 7 salt bridges with basic groups of β subunits [12]. Also in MetHb, IHP is believed to bind to the same site as in deoxyHb [14]. The band of the complex form appeared only in the pH 4.5–6.0 range. From the rate of disappearance of the zone of the liganded state (measured by time-lapse photography), we calculated a half-life for the complex of ~8 min. It should be noted that this has been calculated for the pure complex in the absence of the excess ligand. Even if excess ligand had been present, it would have been completely removed after only 90 s electrophoresis as demonstrated by running the complex albumin–bromophenol blue (BPB) in presence of excess BPB (not shown).

In fig.3B, a similar titration curve for the MetHb–IHS complex is shown. The behaviour is similar to MetHb–IHP complex, except that the pH range of stability of the IHS-liganded form is extended further down in the acidic region, to pH ~3.7 and that the half-life of the complex is reduced by ~0.5 (4 min) as compared to MetHb–IHP. The behaviour of both

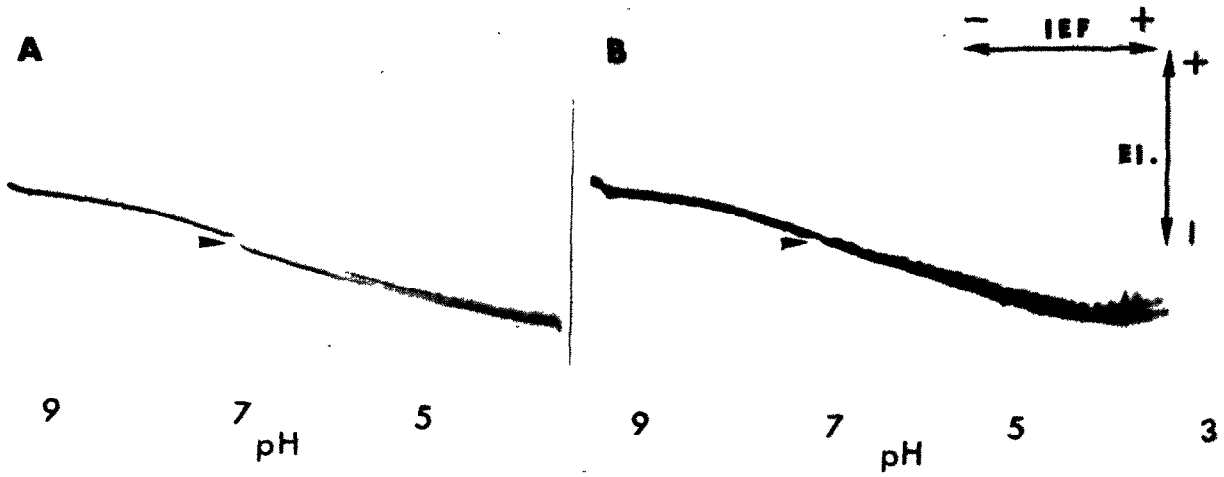

Fig.3. Titration curves of MetHb–IHP complex (A) and MetHb–IHS complex (B). In both cases, the slower moving zones (from the central trough) in the acidic pH region represent liganded Hbs. Blurring of the zones is due to time-dependent loss of ligand, giving rise to a stream of Hb molecules with different mobilities. All other conditions as in fig.1.

complexes is in very good agreement with the chemistry of the Hb binding site. As the most important binding groups appear to be four His in the two β subunits (β2 and 143), it is clear that, as the pH is raised to neutrality and to alkaline, i.e. in regions where His is largely deprotonated, the complex is weakened to such an extent that it cannot exist in absence of excess ligand. Another explanation based on [14] (the pH-dependent allosteric R ↔ T transition) can be given to the observed instability of the complex. Moreover, it has been demonstrated [13] that in going from pH 7.0–7.8, the K_a for binding DPG to deoxyHb is increased by one order of magnitude, from 1.3×10^5 M^{-1} to 1.2×10^4 M^{-1}. Conversely, at pH 4.5, the complex HbA–IHP is again weakened because in going from pH 7–4, the total negative charge of IHP is reduced by 0.5 (from ~8 negative charges to 4). This is not the case with IHS which is a stronger acid than IHP, and the fact that also this complex disappears below pH 3.7 must be due to the well known instability of the Hb molecule itself below pH 4 [2]. Also, the lower half-life of the IHS–MetHb complex, as compared to IHP-liganded HbA, is consistent with the lower charge density of IHS at physiological pH in comparison to IHP [15].

Figure 4A shows the titration curve of HbA complexed with 2,3-DPG. Indeed, only one pH-mobility curve can be seen, indicating that the complex is too short-lived to be separated at any pH by our technique. This is consistent with a K_a for binding DPG to oxyHb of 2.5×10^2 M^{-1} (pH 7.0, 37°C, 130 mM KCl) [16,17]. In fig.4B, we have drawn a titration curve of Hb indicating the pH ranges in which the various complexes are stable.

As the present technique appears to be a valuable tool for the study of protein–ligand interactions, we should like to stress the following aspects:

1. By the present method, it is possible to isolate liganded forms of a protein with a half-life of a minimum of ~3 min. In the case of Hb, this corresponds to a μM order K_a.
2. As the protein is simultaneously titrated from pH 3–10, the pH range of stability of the liganded form can be evaluated. This means that, if the liganded species are stable only at a pH removed from their pI, equilibrium isoelectric focusing will not reveal them. Also, since the half-life of these long-lived intermediates is of the order of a few minutes, equilibrium IEF cannot possibly detect them as there is ample time for the liganded form to be stripped free of ligand.
3. From the shape of the titration curves of the liganded versus the unliganded form it is possible to estimate the amino acid(s) involved in ligand

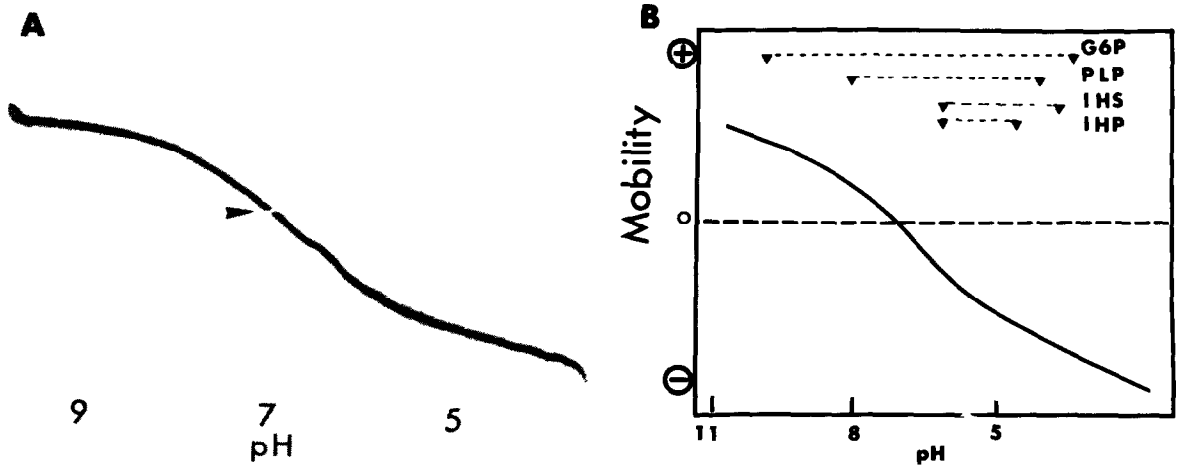

Fig.4. Titration curves of HbA versus HbA–DPG complex (A). Notice that only a single curve can be seen, since the DPG complex is readily split at all pH values. All other conditions as in fig.1. (B) Drawing of a titration curve of Hb with the pH ranges of stability of all the complexes examined.

binding. Thus our data with IHP and IHS clearly suggest a predominant role of His in the binding.

4. As the liganded species moves in a gel zone free of excess ligand, it is possible, by using radioactive ligand, to determine the stoichiometry of the protein–ligand complex.
5. We predict that it should be possible, by the present technique, to isolate and study liganded forms having a much shorter half-life (perhaps in the s or ms order). This could be achieved by blocking the splitting reaction using sub-zero temperatures. This has been elegantly demonstrated [18] where the HbA–ATP complex was isolated by IEF at −10°C. Alternatively, the protein or enzyme could be blocked in a more stable conformation, allowing a much longer life of the liganded state. In the case of Hb, this can be achieved by performing the electrophoresis under anaerobic conditions, since the K_a of organic phosphates to deoxyHb (i.e. to the T structure) is 1000–10 000-fold lower than in the oxy state (R structure) [17]. Alternatively, our present technique could be modified to allow for a constant stream of ligand in the gel.
6. The fact that MetHb–IHP and MetHb–IHS complexes have not been isolated previously by electrophoresis in absence of excess ligand and in the oxyHb state, might be due to the different ionic strength milieu of electrophoresis as opposed to isoelectric focusing media, where the amphoteric ions are kept stationary and the ionic strength reduced to a minimum. In fact, by assuming an Ampholine av. mol. wt 600, an even distribution of ~500 species and the condensation in focused zones occupying ~1% of the original gel volume, we have calculated ~10 mM per each focused Ampholine species. In addition to this, Ampholine molecules are known to have a stabilizing effect on proteins [19].

Acknowledgements

Supported in part by grants 76.01488.04 and CT77.01471.04 from CNR, Roma and by INSERM grants no. 14.75.37 and 41.76.73. P.G.R. thanks INSERM for a short-term fellowship. We thank LKB France for specially providing equipment. The authors are thankful to Dr Gérard Gacon for stimulating discussions.

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